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DELINEATION OF SEVERAL DR-RESTRICTED TETANUS TOXIN T CELL EPITOPES[1]

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We have characterized five human T cell clones specific for tetanus toxin. The combination of different techniques allowed us to precisely map two T cell epitopes within fragments 830–843 and 1273–1284 of tetanus toxin, as formally demonstrated by the use of corresponding synthetic peptides. The three other T cell clones were specific for regions 2–602, 604–742, and 865–1315 of tetanus toxin, respectively.

The five T cell clones were shown to be restricted to HLA-DR Ag. Furthermore, the allele of HLA-DR utilized by the various epitopes has been determined. The use of HLA-DR-transfected L cells as APC directly demonstrated that two epitopes, one of which represented by fragment 1273–1284, were recognized in association with HLA-DRw52a. For the other three T cell epitopes, the data strongly suggested they were recognized in association with HLA-DR5.

Finally, a sixth T cell clone was shown to be specific for tetanus toxoid, the vaccinal preparation of tetanus toxin, and not for other tetanus toxin fragments. This indicated that immunization with tetanus toxoid probably elicits a T cell response directed only in part against native tetanus toxin.

It is now generally accepted that Th lymphocytes recognize soluble protein Ag as processed proteolytic fragments in association with MHC class II molecules on the cell surface of APC (1–3). In recent years, data have been generated indicating that in the murine and in the human systems a limited number of epitopes within a single protein are recognized by T cells in the context of a single MHC class II restriction element (4–13). The basis of these findings has been attributed to the specific binding of these peptides to the proper restriction element (14, 15). Recently, the structure of the HLA-A2 molecule was provided and supports this concept. Portions of the outer extracellular domains of the H chain are arranged so that they could constitute a peptide binding site (16, 17). A similar three-dimensional model has been recently suggested for HLA class II molecules (18).

To date, a limited number of human T cell epitopes have been characterized (5, 19, 20) and only in some cases have data concerning the utilization of the restriction element associated with peptide been provided (20).

Delineation of T cell epitopes and their restriction pattern would be useful not only for understanding the basis of recognition of Ag by class II molecules and TCR but also for designing synthetic vaccines. As recently demonstrated by several authors (21–25), it is possible to elicit neutralizing Ab[4] by coupling B cell epitopes to T cell epitopes.

The recent cloning and sequencing of the t.t. gene and the availability of recombinant bacteria expressing fragments of t.t. of different lengths (26) combined with the fact that almost all human beings are immunized against t.t. make this Ag an ideal choice for such studies.

Here we report the epitope mapping of 6 Th cell clones as well as the determination of the type and allele of MHC class II restriction Ag used. For two of these clones two synthetic peptides of 12–14 amino acid residues have proven to be stimulatory.

MATERIALS AND METHODS

Cells and media for cell culture. The establishment of the four EBV-transformed B cell lines 4·2, 11·3, 8·5, and KK·35 was previously described (27). These cell lines were maintained in culture for the past 2 yr. RPMI medium (GIBCO, Paisley, Scotland) supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 1 mM sodium pyruvate, 5×10^{-5} M 2-ME, and 1% of a 100× mixture of nonessential amino acids (GIBCO, Paisley, Scotland) was used (RPMI-c).

The autologous $CD4^+$ T cell clones KT-1, KT-2, KT-4, KT-30, KT-40, and KT-42 were derived from a single $DR3,5^+$ donor. These T cell clones were established as described (27) and were continuously maintained in culture by periodic restimulation by using irradiated PBMC and 1% PHA, M form (GIBCO, Long Island, NY). After 5 days, the T cell blasts were extensively washed and cultured in RPMI-c supplemented with 40 U/ml of human rIL-2.

Ag preparations and chemicals. t.t., B-fr, and C-fr were obtained from Calbiochem, La Jolla, CA. TT was a gift of Dr. J. Cryz, Swiss Serum Institute, Bern, Switzerland. Protein concentration of these preparations was determined by using a Bio-Rad protein assay (Bio-Rad Laboratories, München, West Germany). A schematic representation of t.t. and derived peptides is presented in Figure 1.

Reduction was performed in PBS (pH 7.0) and 8 M urea by using 20 mM dithiothreitol for 30 min at 37°C. The reduced proteins were carboxymethylated with 80 mM iodoacetamide for 60 min at 4°C in the dark. The material was then extensively dialyzed against water.

Cyanogen bromide treatment was performed as described (28).

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[4] Abbreviations used in this paper: Ab, antibody; t.t., tetanus toxin; B-fr, tetanus toxin B-fragment; C-fr, tetanus toxin C-fragment; RCM-t.t., reduced and carboxymethylated tetanus toxin; CNBr RCM-t.t., cyanogen bromide-treated RCM-t.t.; chym. RCM-C-fr, chymotrypsin-treated RCM-C-fr; chym. RCM-t.t., chymotrypsin-treated RCM-t.t.; TT, tetanus toxoid.

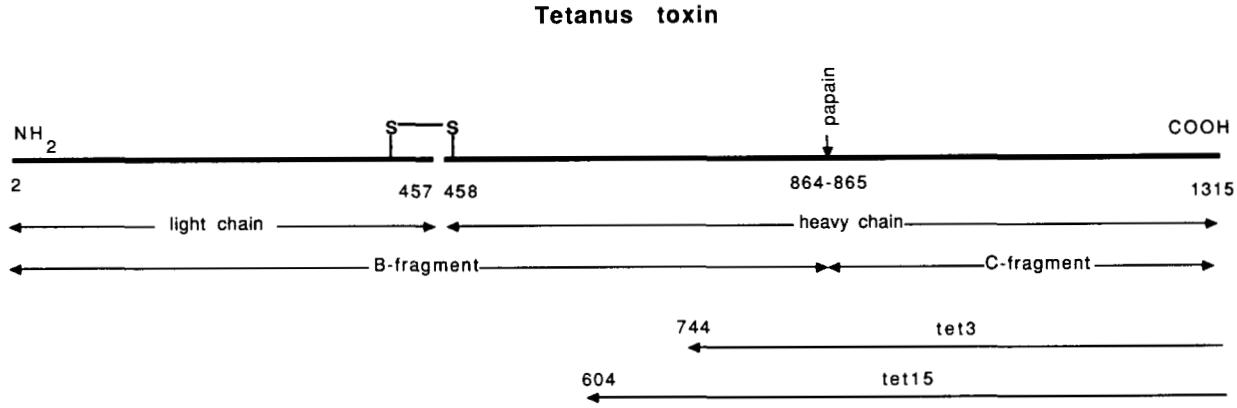

Figure 1. Schematic representation of tetanus toxin. The regions corresponding to the H and L chains, B-fr and C-fr as well as recombinant fragments tet3 and tet15 are shown.

Briefly, lyophilized protein samples were redissolved in 70% formic acid and cyanogen bromide freshly dissolved at 0.1 to 1 mg/ml in acetonitrile was added to a methionine residue molar ratio of 100 to 1. After 24 h incubation at room temperature in the dark, the samples were lyophilized and redissolved in 20 mM NaOH.

Chymotrypsin treatment was performed in PBS (pH 7.0) for 24 h at 37°C by using a weight to weight ratio of enzyme to substrate of 1 to 20 (chymotrypsin, type II, Sigma, St Louis, MO). RCM-t.t. at 2 mg/ml in H_2O was buffered with 10-fold concentrated PBS and chymotrypsin was added to a final concentration of 50 μg/ml. Six hours later, a similar amount of chymotrypsin was added and incubated for further 18 h.

Trypsin (cleavage at the N-terminal side of lysine and arginine residues) treatment was performed in HBSS (GIBCO, Paisley, Scotland) for 24 h at 37°C (trypsin, type XIII, Sigma, St Louis, MO). CNBr RCM-t.t. at 2 mg/ml in 20 mM NaOH was buffered with concentrated HBSS and trypsin was added to a final concentration of 50 μg/ml. Six hours later, a similar amount of trypsin was added and incubated for a further 18 h.

V8 protease treatment (clevage at the N-terminal side of glutamic acid residues at pH 4.0 in ammonium acetate buffer and cleavage at the N-terminal side of glutamic acid and aspartic acid residues at pH 7.8 in phosphate buffer) (29) was performed either in acetate buffer (100 mM ammonium acetate, adjusted at pH 4.5 with acetic acid) or in phosphate buffer (100 mM NaH_2PO_4, adjusted at pH 7.8 with NaOH). The digestion was performed as described for trypsin (*Staphylococcus aureus* V8 endoproteinase, Miles Laboratories, Elkhart, IN).

Lys-C endoproteinase treatment (cleavage at the C-terminal side of lysine residues) was performed in phosphate buffer (100 mM NaH_2PO_4, pH 8.2) as described for trypsin (Lys-C endoproteinase from *Lysobacter enzymogenes*, Calbiochem, La Jolla, CA).

Asp-N endoproteinase of *Pseudomonas fragi* mutant was obtained from Boehringer, Mannheim, West Germany. Digestion (cleavage at the N-terminal side of aspartic acid and cysteic acid residues) was performed in 100 mM NaH_2PO_4 (pH 8.2) for 24 h at 37°C by using a ratio of enzyme to substrate of 1 to 50. CNBr RCM-t.t. at 2 mg/ml in 20 mM NaOH was buffered with 1 M NaH_2PO_4, pH 8.2. Fifty micrograms of CNBr RCM-t.t. peptides were added to 2 μg of lyophilized enzyme. The digestion was performed at 37°C for 24 h.

Performic acid was prepared as described (30). Briefly, 950 μl of 98 to 100% formic acid were mixed with 50 μl 30% H_2O_2 and kept 2 h on ice in a sealed Eppendorf tube. Then, 10 μl of performic acid (oxidization of tyrosine and tryptophane residues) were added to 500 μg CNBr RCM-t.t. at 2 mg/ml and the pH was adjusted to 1 with 10 μl of 37% HCl. After 2 h incubation on ice, the sample was lyophilized.

Lysates of *Escherichia coli* transfected with pUC12 derived plasmids coding for fragments of the t.t. gene were obtained as follows. A single colony was grown overnight at room temperature in 20 ml LB medium (31). Two milliliters of bacteria culture were transferred in 20 ml of fresh LB medium and grown for 1 h at 37°C. Exponentially growing cultures were then induced for 2 h. Tet15 (t.t. fragment 604–1315) under control of the Trp-Lac fusion promoter was induced by adding 1 mM isopropyl β-D-thiogalactopyranoside (Sigma, St Louis, MO). Tet3 (t.t. fragment 744–1315) which is controlled by the tsCI repressor was shifted at 42°C (26). Bacteria were sedimented, resuspended in 2 ml of 30 mM Tris, 20% sucrose, and 10 mM EDTA (pH 8.0) and were treated with 500 μg/ml lysozyme for 30 min at 37°C. Then, the cells were sedimented, resuspended in 2 ml bidistilled H_2O, and sonicated (three times for 20 s, Sonifier B-12, Branson Sonic Power Company, Danbury, CT). Fusion proteins were collected by 50% saturated ammonium sulfate precipitation followed by centrifugation (10,000 × *g*, 30 min, 4°C). The pellet was resuspended in PBS and dialyzed against the same buffer. RCM-tet15 was prepared as described for RCM-t.t.

Immunoprecipitation. t.t. was labeled with ^{125}I according to the procedure described by Greenwood et al. (32). ^{125}I-NaI (600 MBq/μg iodine) was supplied by Amersham International, Amersham, UK. Usually, sp. act. was 5000 cpm/ng protein. One hundred microliters of B cell culture supernatant or RPMI-c were mixed with 5 μl of ^{125}I-t.t. in the presence or absence of 5 μl of cold competitor (t.t., B-fr, or C-fr) added in 100-fold molar excess relatively to labeled t.t. After an incubation of 60 min at room temperature, 20 μl of protein A-Sepharose (Pharmacia, Uppsala, Sweden) were added for an additional 30-min incubation. Then, the beads were washed three times with 500 μl PBS. Beads and pooled supernatants were counted.

ELISA. Aliquots of 100 μl of Ag (5 μg/ml t.t. or tet3, tet15, and RCM-tet15 lysates diluted 100-fold in PBS) were coated overnight at 4°C in wells of 96-well polyvinylchloride plates (Dynatech Laboratories Inc., Alexandria, VA). The wells were then washed three times with 200 μl PBS/0.2% BSA and 100 μl of B cell culture supernatant were incubated for 60 min at room temperature. The wells were then washed as previously and 100 μl of a 1000-fold dilution of goat antibodies anti-human IgG (Sigma, St Louis, MO) in PBS/0.2% BSA were incubated for 60 min. After three repetitive washings, 100 μl of a 1 mg/ml solution of *p*-nitrophenyl phosphate in 1 M diethanolamine and 1 mM $MgCl_2$ (pH 9.8) were added. Ten to 60 min later, absorbance was measured at 405 nm.

Th lymphocyte proliferation assay. One hundred microliters of Ag in RPMI-c were distributed in the wells of 96-(flat or round bottom) well plates (Costar, Cambridge, MA, or Nunc, Roskilde, Denmark, respectively). B cells were washed twice, resuspended in RPMI-c and $2 \cdot 10^4$ fixed (round bottom wells) or live (flat bottom wells) and irradiated B cells (3500 rad) were seeded in 50 μl/well. B cells were fixed as described previously (33). Briefly, B cells were washed once in HBSS and fixed with 0.05% glutaraldehyde for 0.5 min. The reaction was stopped by adding RPMI. The cells were then centrifuged and washed three times.

T cells rested for at least 1 wk in rIL-2 containing RPMI-c were washed twice in RPMI and resuspended in RPMI-c. Two × 10^4 T cells in 50 μl were distributed in the wells. After 48 h incubation at 37°C in 5% CO_2, 1 μCi of [methyl-^{3}H]thymidine (185 GBq/mmol, Amersham International, Amersham, UK) in 10 μl was added. After 20 h pulse, cell-incorporated radioactivity was determined.

It was observed that T cells may proliferate upon addition of CNBr RCM-t.t. and in the absence of B cells. Therefore, in order to establish the MHC restriction, homozygous B cell lines EDR (DR1,1), COX (DR3,3), HAR (DR3,3), WT 20 (DR3,3), WT 49 (DR3,3), LG 14 (DR3,3), ATH (DR5,5), and LG 38 (DR5,5) were pulsed overnight with 40 μg/ml CNBr RCM-t.t., washed three times and $2 \cdot 10^4$ irradiated B cells were tested with $2 \cdot 10^4$ T cells.

Murine L cells transfected with DR3 (TR-166-C2), DRw52a (TR-81·19) and DRw52b (TR-163) were used as APC. The establishment and characterization of these cell lines were described elsewhere (34). After overnight pulsing with 100 μg/ml CNBr RCM-t.t., cells were fixed as described previously and $2 \cdot 10^4$ L cells/well were used in the proliferation assay.

Inhibition of Th lymphocyte proliferation by using anti-MHC class II Ag mAB. KK·35 B cells were pulsed overnight in RPMI-c containing 0.25 μg/ml t.t. and then glutaraldehyde fixed and washed

as described. Inhibition of T cell proliferation by anti-MHC class II Ag mAb was observed only with a limiting number of t.t. pulsed and fixed B cells; $5 \cdot 10^2$ to $5 \cdot 10^3$ fixed B cells were added to $2 \cdot 10^4$ T cells to a 100 μl final volume of RPMI-c. mAb L-243 (anti-DR mAb, American Tissue Collection, Rockville, MD) (35), B-7.21 (anti-DP mAb, Dr. R. Accolla, Ludwig Institute, Epalinges, Switzerland) (36, 37) and Leu-10 (anti-DQ mAb, Becton Dickinson, Münchenstein, Switzerland) were used at a final concentration of 8 μg protein/ml.

Purification, amino acid analyses and synthesis of peptides. Chym. RCM-C-fr was chromatographed on a Vydac 10 C-18 reverse phase column (HPLC technology Ltd., Palos Verdes Peninsular, CA) by using a Waters Associates System (Milford, MA). Elution was performed by forming a linear gradient of 0% to 30% acetonitrile (Romil Chemicals, Shepshed Leics, England) and either 25 mM ammonium acetate (pH 6.0) or 0.05% trifluoroacetic acid. Usually the gradient was completed in 60 min at a flow rate of 1 ml/min. One milliliter fractions were collected, lyophilized, and resuspended in water and subsequently tested in a T cell proliferation assay.

Peptides were hydrolyzed in 6 N HCl under vacuum and the amino acid composition was determined by the dabsyl chloride method (38).

Peptides were sequenced on an automated sequencer (Applied Biosystems, Foster City, CA) by using the sequential Edman degradative method (39).

Synthetic peptides were obtained by solid phase synthesis according to the method of Merrifield modified by Atherton et al. (40) by using F-MOC protected amino acids (Bachem, Bubendorf, Switzerland).

Peptides were sequentially purified by gel filtration on a Sephadex G-10 (Pharmacia AB, Uppsala, Sweden) column and then reverse phase HPLC by using a C-18 column as described above.

RESULTS

Characterization of t.t.-specific B cell lines. To take full advantage of the high presenting capacity of Ag-specific B cells, we have characterized the binding specificity of the Ab they produced.

Radioiodinated t.t. was immunoprecipitated with Ab secreted by the various B cell lines in the presence or absence of an excess of either t.t., B-fr, or C-fr. In the presence of 100-fold molar excess of cold t.t. relatively to labeled t.t., the binding was completely abrogated (Table I). When B-fr was used as competitor, ^{125}I-t.t. binding by 8·5, 11·3, and KK·35 Ab was not reduced whereas ^{125}I-t.t. binding by 4·2 Ab was reduced by 80%. On the other hand, unlabeled C-fr added in excess inhibited binding of ^{125}I-t.t. by 8·5, 11·3, and KK·35 Ab, whereas it did not affect binding of 4·2 Ab (Table I). Taken together, these data indicated that 8·5, 11·3 and KK·35 Ab bound determinants located on the C-fr whereas 4·2 Ab was specific for the B-fr.

An ELISA was used to assess that the C-fr containing recombinant fragments 744-1315 (tet3) and 604-1315 (tet15) were detected by Ab produced by the t.t.-specific B cells (Fig. 1). The 8·5, 11·3, and KK·35 Ab detected tet3 and tet15 fusion proteins when adsorbed on a plastic solid-phase, whereas the B-fr-specific 4·2 Ab did not detect them (Table II, Expt. 1). RCM-tet15 was no more bound by 8·5 and 11·3 Ab (Table II, Expt. 2). These data indicated that tet3 and tet15 t.t. recombinant fragments were recognized by the three C-fr-specific Ab and that the binding of these Ab was dependent on the conformation of the determinants they recognized.

Mapping of T cell epitopes by using proteolytic or recombinant fragments of t.t. Different t.t. preparations (Fig. 1) were tested for their ability to trigger the proliferative response of six human T cell clones.

Out of six T cell clones examined, three of them were specific for the B-fr and two others for the C-fr (Table III, Expt. 1). The clone KT-1 was not stimulated to proliferate upon challenge with either t.t., B-fr, or C-fr. However, this clone was specific for TT, indicating that it recognized either an epitope specific for TT or a clostridial protein found in TT preparations (Table III, Expt. 1). Two recombinant fragments of t.t. were used to further define the location of the epitopes recognized by the T cell clones (26). Tet3 and tet15 were recognized by the Ab secreted by the C-fr-specific B cell lines 8·5, 11·3, and KK·35 (Table II). Tet3 covers the region 744–1315 and tet15 the region 604–1315 of t.t. (Fig. 1). As expected, KT-4 and KT-30 T cells which are specific for the C-fr recognized tet3 and tet15 fusion proteins presented by KK·35 B cells (Table III, Expt. 2).

KT-2 cells recognized tet3 and tet15, KT-42 only tet15 and KT-40 none of them (Table III, Expt. 2). Therefore, KT-2 recognized an epitope located on the overlapping fragment between the B-fr and tet3 which is the peptide 744–864. KT-42 was specific for the fragment 604–743, representing the N-terminal region of tet15 which does not overlap with tet3. KT-40 recognized an epitope located on the fragment 2–602.

The effect of Ag specificity of the B cells on their presenting capacity was analyzed. Proliferation of the KT-2 T cell clone was measured in response to t.t. and B-fr presented by either the B-fr-specific B cell line 4·2 or the C-fr-specific B cell line KK·35. Whatever was the presenting cell, KT-2 T cell proliferation triggered by t.t. was similar (Fig. 2). In contrast, 4·2 B cells stimulated KT-2 T cells with B-fr at a 100-fold lower concentration

TABLE I

Binding of t.t., B-fr, and C-fr by Ab produced by t.t.-specific B cell lines

B Cell Culture Supernatant[a]	^{125}I-t.t., cpm × 10^{-3} bound (% inhibition)[b], with Cold Ag Competitor:			
	None	t.t.	B-fr	C-fr
8·5	10.3	1.2 (87)	9.3 (6)	0.9 (89)
11·3	8.5	0.5 (95)	8.3 (2)	1.5 (83)
KK·35	9.0	0.5 (95)	8.7 (0)	0.8 (91)
4·2	6.3	0.8 (87)	1.1 (80)	6.2 (0)
Medium	0.3			

[a] Five microliters of ^{125}I-t.t. were added to 100 μl of B cell culture supernatant or medium in the presence or absence of unlabeled t.t., B-fr, or C-fr added 100-fold in molar excess relative to ^{125}I-t.t. After 60 min, 20 μl of protein A-Sepharose were added for 30 min.

[b] Washed beads and supernatants were counted. Results are the mean of triplicates. SD were less than 10%. Percentage of inhibition due to the presence of cold Ag competitor is indicated in parentheses.

TABLE II

Immunodetection of t.t. recombinant fragments tet3 and tet15 by C-fr-specific Ab

B Cell Culture Supernatant[a]	Absorbance at 405 nm[b]			
	t.t.	tet3	tet15	RCM-tet15[c]
Expt. 1				
KK·35	0.75	0.50	0.57	ND
8·5	0.76	0.47	0.53	ND
4·2	0.80	0.18	0.19	ND
None	0.09	0.19	0.17	ND
Expt. 2				
8·5	1.46	1.35	1.41	0.37
11·3	1.62	1.27	1.44	0.31
None	0.12	0.18	0.27	0.30

[a] One hundred microliters of B cell culture supernatant or RPMI-c were incubated for 60 min and washed as previously. One hundred microliters of a 1000-fold dilution of goat Ab anti-human IgG coupled to alkaline phosphatase were incubated for 30 min and then washed three times.

[b] Absorbance of a 1 mg/ml solution of *p*-nitrophenyl phosphate was measured at 405 nm after 10 to 60 min. The results are the mean of quadriplicate wells. SD were less than 10%.

[c] Wells were coated overnight at 4°C with t.t. (5 μg/ml), tet3, tet15 and RCM-tet15 (lysate dilution 1/100 in PBS) and then washed (3 × 200 μl PBS/0.2% BSA).

TABLE III

Proliferative response of TT-specific T cell clones to various t.t. preparations

Ag[a]	[^{3}H]Thymidine, cpm × 10^{-3}, Incorporated[b]					
	KT-1	KT-2	KT-4	KT-30	KT-40	KT-42
Expt. 1						
TT	19.4 ± 2.1	**31.0 ± 4.5**	**44.5 ± 1.2**	**27.4 ± 3.2**	**45.6 ± 7.2**	**45.7 ± 2.9**
t.t	7.7 ± 0.3	**48.1 ± 5.3**	**51.7 ± 5.3**	**55.5 ± 3.3**	**105.1 ± 3.4**	**49.8 ± 3.2**
B-fr	7.3 ± 0.3	**23.2 ± 4.8**	4.2 ± 0.1	3.9 ± 0.7	**99.2 ± 4.9**	**43.3 ± 5.2**
C-fr	4.3 ± 0.2	2.8 ± 0.2	**48.0 ± 7.1**	**68.2 ± 4.5**	9.1 ± 1.2	6.0 ± 0.4
None	7.2 ± 0.4	2.0 ± 0.1	4.1 ± 0.3	3.0 ± 0.1	5.5 ± 1.1	6.2 ± 1.2
Expt. 2						
t.t.	ND	**17.3 ± 2.1**	**9.8 ± 1.3**	**18.3 ± 2.1**	**30.8 ± 2.2**	**21.6 ± 1.9**
tet3	ND	**13.3 ± 1.9**	**6.2 ± 1.1**	**15.2 ± 1.7**	0.6 ± 0.1	1.6 ± 0.1
tet15	ND	**15.0 ± 0.8**	**5.4 ± 1.1**	**14.4 ± 1.2**	0.5 ± 0.1	**11.8 ± 1.1**
None	ND	1.3 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	0.8 ± 0.1	0.9 ± 0.1

[a] Aliquots of $2 \cdot 10^4$ irradiated KK·35 B cells (3500 rad) were added to $2 \cdot 10^4$ T cells in the presence or absence of 1 μg/ml of various t.t. preparations (Expt. 1) in a final volume of 200 μl RPMI-c. In the case of B-fr, 4·2 B cells were used (Expt. 1). In Expt. 2, KK·35 B cells were used and proliferative responses obtained with 1/1000 bacterial lysate dilution are presented.

[b] After 2 days of incubation, 1 μCi[^{3}H]thymidine was added in each culture. Twenty-four hours later, incorporated radioactivity was determined. The results are expressed in cpm ± 1 SD for quadriplicate wells. **Bold numbers** indicate significant T cell proliferation. In the absence of Ag, no T cell proliferation was observed either in the presence of KK·35 or 4·2 B cells. In Expt 1, background values for KK·35 and 4·2 B cells alone were 5.5 ± 1.4 and 2.3 ± 1.1, respectively. In Expt. 2, background value for KK·35 was 1.1 ± 0.2.

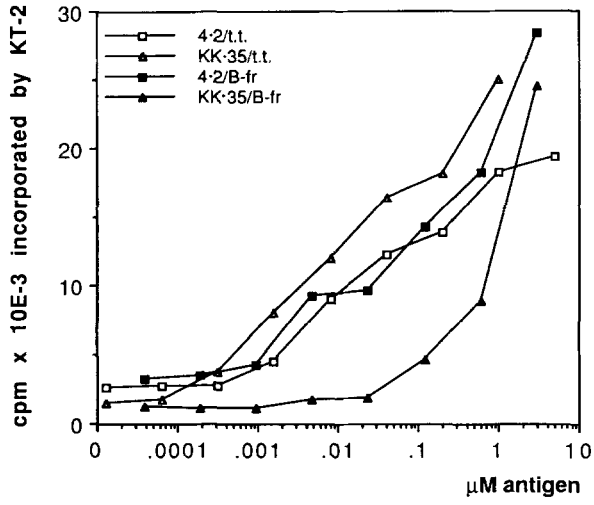

Figure 2. Proliferative response of KT-2 T cell clone to t.t. or B-fr presented by B-fr- or C-fr-specific B cell line. Aliquots of $2 \cdot 10^4$ KT-2 T cells were added to $2 \cdot 10^4$ irradiated (3500 rad) B-fr-specific B cells (4·2, *square*) or C-fr-specific B cells (KK·35, *triangle*) in the presence of various concentrations of t.t. (*open symbol*) or B-fr (*closed symbol*) in a final volume of 200 μl of RPMI-c. After 2 days of incubation, 1 μCi [^{3}H] thymidine was added to each culture. Twenty-four hours later, incorporated radioactivity was determined. The results are expressed in cpm for quadriplicate wells. SD were less than 20%.

than KK·35 B cells (Fig. 2). These observations indicated that Ag presenting efficiency was greatly enhanced upon recognition of Ag by B cell surface Ig.

Delineation of antigenic peptides using proteolytic digestion and chemical modifications of tetanus toxin. It was observed that glutaraldehyde fixed B cells could present a preparation of CNBr RCM-t.t. to the five T cell clones but not the intact either native or reduced and alkylated protein (Table IV, data not shown). Therefore, the epitopes of these T cell clones were all located between methionine residues. The effect of several enzymatic or chemical modifications on the antigenicity of CNBr RCM-t.t. preparation was then evaluated. It was assumed that loss of antigenicity implies that the hit amino acid residues are present in the T cell epitope. Alternatively, if the modification did not affect the T cell response these residues are absent from the epitope.

CNBr RCM-t.t. preparations were treated with trypsin, Lys-C endoproteinase, V8 protease at pH 4.0 in ammonium acetate buffer and at pH 7.8 in phosphate buffer, Asp-N endoproteinase, chymotrypsin, and performic acid.

T cell proliferation was measured in response to various concentrations of these CNBr RCM-t.t. preparations presented by glutaraldehyde-fixed B cells. This procedure was done to ensure that the results would not be affected by the presence of some unmodified t.t. material in the various preparations. The comparison of the concentration of the various CNBr RCM-t.t. preparations required to elicit 50% of the maximal proliferative response relatively to the untreated preparation allowed one to conclude if lysine, aspartic acid, glutamic acid and eventually tyrosine and/or tryptophane residues were present or absent in the antigenic peptides. This set of experiments are presented in Table IV. Subsequently, the peptides which fitted to these conditions were sought within the t.t. sequence.

The epitope of the KT-2 T cell clone contained at least one lysine residue and no aspartic acid and glutamic acid residues (Table IV). The t.t. sequences 830–843 and 849–864 matched these conditions. Therefore, synthetic peptides corresponding to these two fragments were produced and tested in a proliferation assay. The peptide 830–843 induced KT-2 T cells to proliferate, whereas peptide 849–864 did not (Fig. 3*a*). Consequently, the t.t. fragment 830–843 contains the epitope recognized by KT-2 T cells. The amino acid sequence of KT-2 antigenic peptide is presented in Figure 3*a*.

It was observed that KT-4 T cell proliferation could be induced by preparations of chym. RCM-t.t. presented by glutaraldehyde-fixed B cells (Table IV). This indicated that a fragment bearing KT-4 T cell epitope was produced. The peptide which was antigenic for KT-4 T cells was purified. A preparation of chym. RCM-C-fr was submitted to two steps of reverse phase high pressure liquid chromatography (see *Material and Methods* for procedure followed). Due to the uncertainty of the N-terminal residue, peptides corresponding to sequences 1272–1284 and 1273–1284 with either an aspartic acid or an asparagine residue in position 1272 were produced. These peptides proved to be equally stimulatory (Fig. 3*b* and

TABLE IV

KT-2, KT-4, KT-30, KT-40, and KT-42 T cell proliferative responses to various chemically modified t.t. preparations

Treatment of CNBr RCM-t.t[a]	CNBr RCM-t.t. (μg/ml) Required to Elicit 50% of Maximal T Cell Proliferative Response[b]				
	KT-2	KT-4	KT-30	KT-40	KT-42
—	15	25	2	25	2
Tryps.	>100	>100	>100	>100	>100
Lys-C	>100	25	>100	>100	>100
V8, pH 4.0	5	25	3	15	15
V8, pH 7.8	10	>100	2	>100	>100
Asp N	5	>100	10	>100	>100
Chym.	>100	20	>100	>100	>100
Perf. acid	15	25	30	>100	6
Present[c]	Lys	Asp	Lys	Lys, Asp, Trp/Tyr	Lys, Asp, Glu
Absent	Asp, Glu	Lys, Glu	Asp, Glu	Glu	

[a] CNBr RCM-t.t. (—), trypsin-treated CNBr RCM-t.t. (Tryps.), Lys C endoproteinase-treated CNBr RCM-t.t. (Lys-C), V8 endoproteinase-treated CNBr RCM-t.t. at pH 4.0 (V8, pH 4.0), V8 endoproteinase-treated CNBr RCM-t.t at pH 7.8 (V8, pH 7.8), Asp N endoproteinase-treated CNBr RCM-t.t (Asp N), chymotrypsin-treated RCM-t.t. (Chym.), and performic acid treated CNBr RCM-t.t. (Perf. acid) were prepared as described in *Materials and Methods*.

[b] T cells ($2 \cdot 10^4$) were added to $2 \cdot 10^4$ glutaraldehyde-fixed KK·35 B cells in the presence of various concentrations of t.t. preparations. After 2 days of incubation, 1 μCi [^{3}H]thymidine was added in each culture. Twenty-four hours later, incorporated radioactivity was determined. The results represent the concentrations of t.t. preparations in μg/ml required to elicit 50% of the maximal proliferative response.

[c] "Present" and "absent," respectively, mean that the indicated amino acid residues are found and are not found in the corresponding T cell epitope.

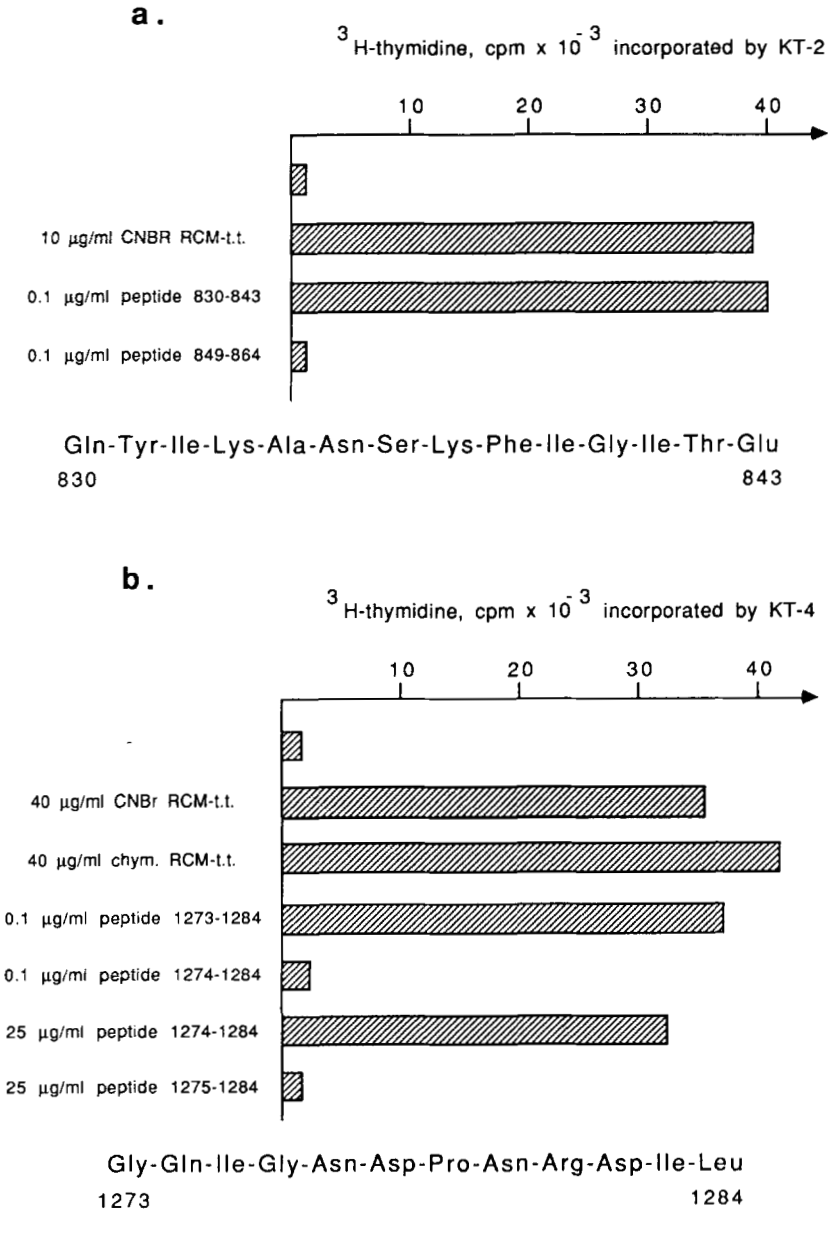

Figure 3. KT-2 and KT-4 T cell proliferative response to t.t. preparations and synthetic peptides. *a*, $2 \cdot 10^4$ irradiated B cells (3500 rad) were added to $2 \cdot 10^4$ KT-2 T cells in the presence or absence of either 10 μg/ml CNBr RCM-t.t., 0.1 μg/ml peptide 830–843 or 0.1 μg/ml peptide 849–864 in a final volume of 200 μl RPMI-c. The t.t. sequence 830-843 is presented with the standard three-letter code. *b*, $2 \cdot 10^4$ irradiated B cells (3500 rad) were added to $2 \cdot 10^4$ KT-4 T cells in the presence or absence of either 40 μg/ml CNBr RCM-t.t., 40 μg/ml chymotrypsin-treated RCM-t.t., 0.1 μg/ml peptide 1273–1284, 0.1 μg/ml peptide 1274–1284, 25 μg/ml peptide 1274–1284, or 25 μg/ml peptide 1275–1284 in a final volume of 200 μl RPMI-c. The t.t. sequence 1273–1284 is presented with the standard three-letter code. After 2 days of incubation, 1 μCi [^{3}H] thymidine was added in each culture. At 24 h, incorporated radioactivity was determined. The results are expressed in cpm for quadriplicate wells. SD were less than 20%.

data not shown). This demonstrates that the KT-4 T cell epitope is located within peptide 1273–1284 of t.t. The amino acid sequence of this peptide is presented in Figure 3*b*. Shorter synthetic peptides were also produced. Peptide 1274–1284 was 250-fold less stimulatory than peptide 1273–1284. Half-maximal response was triggered by 20 ng/ml peptide 1273–1284 and by 5 μg/ml peptide 1274–1284. Peptide 1275–1284 was not at all antigenic for KT-4 T cells (Fig. 3*b*).

Determination of the MHC class II restriction elements and alleles utilized by the T cell clones KT-2, KT-4, KT-30, KT-40, and KT-42. T cells were stimulated to proliferate by using a limited number of t.t. pulsed and fixed B cells. Either anti-DR, -DP, or -DQ mAb were added in culture to determine which of these three MHC class II elements was used. Anti-DR mAb strongly inhibited (85 to 94%) the proliferation of the five T cell clones tested. Anti-DP and anti-DQ mAb were ineffective in inhibiting proliferation of the T cell clones analyzed (Table V). When the number of B cells was increased ($2 \cdot 10^4$ instead of 500 to 5000), no inhibition was observed with anti-DR mAb, ruling out the possibility that this mAb may be toxic for T cells (data not shown). Thus, we concluded that DR Ag is the restriction element used by these T cell clones.

The clones were then tested for their ability to proliferate by using different CNBr RCM-t.t. pulsed DR homozygous B cell lines as APC. KT-4 and KT-42 T cell clones were stimulated to proliferate upon interaction with CNBr RCM-t.t. pulsed COX and HAR and not with WT-20, WT-49, and LG-14 DR3 homozygous B cell lines (Table VI).

KT-2, KT-30, and KT-40 T cell clones proliferative responses were induced by CNBr RCM-t.t. pulsed ATH and LG 38 DR5 homozygous B cell lines (Table VI). The EDR DR1 homozygous B cell line did not present CNBr RCm-t.t. peptides to any of the T cell clones (Table VI).

The observation that KT-4 and KT-42 T cells were not stimulated by Ag pulsed WT-20, WT-49, and LG-14 cells suggested that DRα:DR3βIII was the restriction element utilized by these two T cell clones. To test this hypothesis, L cells transfected with the genes coding for DR3, DRw52a, or DRw52b were used as APC (34). TR-166 C2, TR-81·19, and TR-163 L cells, respectively, expressed 40, 95, and 15% of HLA-DR Ag compared with KK·35 B cells as assessed by L.243 mAb binding assay (data not shown). TR-81·19 L cells (DRw52a) pulsed with CNBr RCM-t.t. and glutaraldehyde fixed induced KT-4 and KT-42 T cell proliferative responses. Neither TR-166-C2 L cells (DR3) nor TR-163 L cells (DRw52b) displayed this capacity (Table VII). Therefore, DRw52a is the restriction element utilized by KT-4 and KT-42 T cell clones.

DR5 allele is always associated with DRw52b within the Caucasian population (41). In all experimental conditions tested, KT-2, KT-3, or KT-40 T cells were not stimulated by Ag-pulsed TR163 L cells (data not shown). Thus, although not formally proven, it is reasonable to assume that DR5 is the restriction element utilized by KT-2, KT-30, and KT-40 T cell clones.

Table VIII summarizes the data obtained regarding the epitopes recognized and the MHC class II molecules utilized by the various T cell clones.

DISCUSSION

In the present study, t.t.-specific human T cell clones were characterized in terms of their Ag specificity and class II restriction element utilized. Although all the restriction elements used were determined (Table VIII), the exact antigenic fragment recognized by the T cells was established for only two clones (Table VIII). Although the number of T cell clones analyzed was small, no immunodominant site was detected. In consequence, it is thought that other T cell epitopes could be detected through the entire sequence of t.t. Another T cell clone was shown to be specific for TT but not for native t.t. This T cell clone could recognize clostridial proteins found in the crude preparations of TT which contain about 50% of other bacterial proteins (Dr. J. Cryz, Swiss Serum Institute, Bern, Switzerland, personnal communication). Alternatively, it may recognize an epitope created by formaldehyde treatment of t.t. Upon detoxification, some lysine residues are methylated and cross-linked with tyrosine and other lysine residues (42). Preliminary results indicate that this clone is not an isolated case. it would mean that vaccination with TT elicits a T cell response which is directed only in part against the native form of t.t.

Fusion proteins containing t.t. fragments were used to further define the location of T cell epitopes on t.t. This technique was readily applicable in our system without any purification step since some of the fusion proteins displayed a native structure. Tet3 and tet15 fusion proteins were recognized by C-fr-specific Ab whereas reduction and alkylation abrogated binding. Therefore, with C-fr-specific B cells, lysate of 10^6 bacteria was sufficient to trigger T cell proliferation, whereas if fusion protein unspecific B cells were used, 10,000-fold more lysate would be required. The latter dilution of bacteria lysate (10^{10} bacteria) appeared toxic in the T cell proliferation assay.

TABLE V

Blocking of proliferative response of t.t.-specific T cell clones by mAb directed against various MHC class II molecules

mAb[a]	[^{3}H]Thymidine, cpm × 10^{-3} incorporated[b]				
	KT-2	KT-4	KT-30	KT-40	KT-42
None	8.9 ± 1.1	15.2 ± 2.0	10.4 ± 0.5	19.2 ± 0.8	56.0 ± 2.7
L.243 (anti-DR)	**0.5 ± 0.1 (94)**	**2.3 ± 0.5 (85)**	**1.2 ± 0.1 (88)**	**1.1 ± 0.3 (94)**	**4.2 ± 1.1 (93)**
Leu 10 (anti-DQ)	6.4 ± 1.1 (28)	14.3 ± 1.9 (6)	11.3 ± 0.2 (−9)	15.0 ± 2.4 (22)	ND
B7.21 (anti-DP)	8.3 ± 1.3 (7)	15.8 ± 2.2 (−4)	10.4 ± 1.1 (0)	14.6 ± 2.1 (24)	ND

[a] KK·35 B cells were pulsed overnight with 0.25 μg/ml t.t. and then glutaraldehyde fixed. Aliquots of $5 \cdot 10^3$ B cells (500 in the case of KT-42 T cells) were added to $2 \cdot 10^4$ T cells in the presence or absence of 8 μg/ml mAb L.243 (anti-DR), mAb Leu 10 (anti-DQ), or mAb B 7.21 (anti-DP) in a final volume of 200 μl RPMI-c.

[b] After 2 days of incubation, 1 μCi[^{3}H]thymidine was added in each culture. Twenty-four hours later, incorporated radioactivity was determined. The results are expressed in cpm ± 1 SD for triplicate wells. **Bold numbers** indicate significant inhibition of T cell proliferation. Percentage of inhibition relatively to control without mAb is indicated in parentheses.

TABLE VI

Presentation of CNBr RCM-t.t. peptides to KT-2, KT-4, KT-30, KT-40, and KT-42 T cell clones by various HLA-DR homozygous B cell lines

B Cell Line[a]	[^{3}H]Thymidine, cpm × 10^{-3} Incorporated[b]					
	KT-2	KT-4	KT-30	KT-40	KT-42	-
KK·35 (3,5)	**85.9 ± 20.3**	**113.5 ± 1.3**	**18.9 ± 2.6**	**32.3 ± 4.0**	**22.3 ± 0.8**	7.1 ± 0.2
EDR (1,1)	2.0 ± 0.1	2.4 ± 0.3	1.9 ± 0.1	2.3 ± 0.3	2.5 ± 0.1	2.2 ± 0.2
HAR (3,3)	2.3 ± 0.7	**127.7 ± 7.9**	1.7 ± 0.3	1.7 ± 0.6	**20.4 ± 0.8**	2.1 ± 0.1
COX (3,3)	1.4 ± 0.1	**108.5 ± 2.0**	0.9 ± 0	1.3 ± 0.3	**14.9 ± 0.5**	1.2 ± 0.1
WT-20 (3,3)	1.1 ± 0.2	1.0 ± 0.2	0.6 ± 0.1	0.9 ± 0.3	0.9 ± 0.1	0.8 ± 0.2
WT-49 (3,3)	0.6 ± 0	1.0 ± 0.2	0.3 ± 0.1	0.9 ± 0.3	0.5 ± 0.1	0.4 ± 0.2
LG-14 (3,3)	9.7 ± 0.8	7.2 ± 0.3	6.9 ± 0.1	7.9 ± 0.4	8.0 ± 1.2	10.3 ± 0.7
ATH (5,5)	**77.1 ± 5.3**	1.7 ± 0	**12.5 ± 2.9**	**18.5 ± 4.6**	1.4 ± 0.1	1.5 ± 0.1
LG-38 (5,5)	**81.4 ± 1.4**	2.3 ± 0	**16.8 ± 0**	**33.0 ± 3.2**	1.9 ± 0.1	2.7 ± 0
-	0.2 ± 0.1	0.2 ± 0.1	0.5 ± 0.5	0.4 ± 0.4	0.1 ± 0	0.1 ± 0.1

[a] B cells were pulsed overnight with 40 μg/ml CNBr RCM-t.t. and then washed three times. Aliquots of $2 \cdot 10^4$ irradiated B cells (3500 rad) were added to $2 \cdot 10^4$ T cells in a final volume of 200 μl RPMI-c.

[b] After 2 days of incubation, 1 μCi[^{3}H]thymidine was added in each culture. Twenty-four hours later, incorporated radioactivity was determined. The results are expressed in cpm ± 1 SD for quadriplicate wells. **Bold numbers** indicate significant T cell proliferation.

TABLE VII

Presentation of CNBr RCM-t.t. peptides to KT-4 and KT-42 T cell clones by KK·35 B cells and various HLA-DR transfected L cells

APC[a]	[^{3}H]Thymidine, cpm × 10^{-3} Incorporated[b]	
	KT-4	KT-42
KK·35	**27.3 ± 1.4**	**110.1 ± 1.3**
TR-166-C2 (DR3)	0.2 ± 0.1	0.2 ± 0.1
TR-81-19 (DRw52a)	**9.7 ± 2.7**	**16.6 ± 5.6**
TR-163 (DRw52b)	0.2 ± 0.1	0.2 ± 0.1
-	0.1 ± 0.1	0.1 ± 0.1

[a] KK·35 B cells and L cells were pulsed overnight with 100 μg/ml CNBr RCM-t.t. and then glutaraldehyde fixed. Aliquots of $2 \cdot 10^4$ B cells or L cells were added to $2 \cdot 10^4$ T cells in a final volume of 200 μl RPMI-c.

[b] After 2 days of incubation, 1 μCi[^{3}H]thymidine was added in each culture. Twenty-four hours later, incorporated radioactivity was determined. The results are expressed in cpm ± 1 SD for quadriplicate wells. **Bold numbers** indicate significant T cell proliferation. KT-4 and KT-42 T cell proliferation triggered by unpulsed APC was less than 0.5 × 10^3 cpm.

TABLE VIII

Summary of t.t. fragments recognized by the various T cell clones and the restriction elements utilized

T Cell Clone	Shortest Defined t.t. Fragment Containing T Cell Epitope	Restriction Element
KT-2	830–843	DR5
KT-4	1273–1284	DRw52a
KT-30	865-1315	DR5
KT-40	2–602	DR5
KT-42	604–742	DRw52a

The use of specific endopeptidases to map the epitope recognized by T cell clone KT-2 could represent a general approach for delineating epitopes in cases in which the protein is available in μg quantities. This novel and simple method has the advantage of limiting the number of peptides to be synthesized. For example, in the case of this epitope only two peptides were made (Fragments 830–843 and 849–864). In contrast, according to the method established by the group of Atassi (6), 10 overlapping peptides of 15 amino acids had to be synthesized to cover the entire sequence 744–864 of t.t. The peptide 830–843 contains one of the two sequence patterns which Rothbard and Taylor (43) recently described as being common to numerous T cell epitopes. The algorithm established by De Lisi and Berzofsky (44) also predicted that fragment 830–843 might represent a T cell epitope. This peptide could assume an α-helical structure segregating hydrophobic and hydrophilic residues. On the other hand, epitope 1273–1284 does not contain any of the motifs described above. The presence of a proline residue in the middle of the KT-4 epitope suggests the possibility that this peptide does not display an α-helical structure partitioning hydrophobic and hydrophilic residues. This peptide might assume a β-turn structure as proline and asparagine are two of the seven most frequent residues found in loops (45). Furthermore, aspartic acid 1278 and arginine 1281 may be important for stabilizing the hairpin stucture through an ionic bond. A similar structure was already suggested for another T cell epitope in the mouse (11).

The Ag specificities of three other clones were partially characterized and shown to be different. As KT-4, KT-30 was specific for C-fr but was not stimulated by the t.t. fragment 1273–1284. Therefore, the epitopes recognized by these two clones are different. The Ag specificity of clone KT-42 was assigned within fragment 604–742. However, we did not succeed in precisely mapping the KT-42 epitope even though all the peptides containing lysine, aspartic acid and glutamic acid residues within fragment 604–742 were produced. One explanation is that asparagine or glutamine residues may have been deamidated during t.t. purification, suggesting that enzymatic cleavage with V8 protease and/or Asp-N endoproteinase may have occurred at these modified sites. The conversion of asparagine and glutamine residues to the corresponding acid is a well known and common modification occuring in proteins (46). An example of deamidation was also observed in the natural peptide 1272–1284 in which an aspartic acid residue at position 1272 was found by sequence analysis in place of an asparagine residue as determined by DNA sequence analysis (data not shown).

Finally, KT-40 T cell clone recognized an epitope located in the t.t. fragment 2–602. Preliminary results indicated that this T cell clone was specific for the light chain (data not shown).

The five T cell clones studied were DR-restricted. This observation was not surprising since several reports have indicated that DR antigen is the most frequently used restriction element (47, 48). Furthermore, the five T cell clones have different antigenic specificities. Although the number of T cell clones analyzed was small, the observations reported here would indicate the absence of any dominant epitopes in the t.t. protein in contrast to what observed in several murine systems (2, 3, 7, 11) and once

in the human system (5).

Three of these clones are DR5-restricted although this was not formally proven. On the other hand, we have directly demonstrated that two t.t.-specific T cell clones utilize DRw52a molecules as restriction element by the use of murine L cells transfected with HLA class II molecules. Furthermore, the epitope of KT-4 which is associated with DRw52a was precisely mapped within a peptide of 12 amino acid residues. In this respect, Irlé et al. (49) have recently shown that DR3, DRw52a, and DRw52b represent functional restriction elements for TT-specific T cell clones. However, they did not define TT sequences recognized by these T cell clones.

Tiercy et al. (41) have shown that the allele DR3 is always associated with DRw52a or DRw52b in the Caucasian population. This would explain why out of five DR3 homozygous B cell lines, three failed to present CNBr RCM-t.t. peptides to KT-4 and KT-42 T cells. Typing showed that WT-49 B cell line is DR3, DRw52b (C. Irlé, Transplantation Immunology Unit, Hôpital Cantonal Universitaire, Geneva, Switzerland, personal communication). Subsequently, we postulate that WT-20 and LG-14 B cell lines also express DRw52b. It is noteworthy to mention that T cell clones which share the same antigenic specificity with KT-4 clone are found in other DR3 and DRw6 individuals (P. Panina, A. Tan, S. Demotz, G. Corradin, and Lanzavecchia, manuscript in preparation). These two haplotypes are associated with DRw52a (41).

The sequence of the genes coding for DR3, DRw52a, and DRw52b have been recently published (49). DRw52a and DRw52b differ in 11 amino acid residues. These differences seem to be mainly located in the cleft of the HLA class II molecule which represents the putative peptide binding site (18).

It is now conceivable to use KT-4 and KT-2 antigenic peptides as carrier molecule for B cell epitopes in DRw52a and DR5 positive and TT-primed individuals in a manner similar to that described recently by several authors (21–25). The use of peptide as carrier would in principle avoid the manifestation of carrier specific suppression encountered in different carrier systems when the entire molecule is used (50–52).

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